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Ahiflower seed and its press cake as sources of nutrients for laying hens and omega-3 fatty acids in their eggs

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ABSTRACT

240 64-week-old Lohman LSL-Lite laying hens were used to evaluate the effect of ahiflower seed (AS) and its press cake (APC) on egg yolk fatty acid profile, production performance, apparent total tract nutrient digestibility (ATTD), egg quality, eggshell mineral content, and fecal microbiota composition for 12 weeks in a completely randomized design, with 6 replicates of 5 birds in a cage. The diets included a control (CD), CD supplemented with 10 % flaxseed (FS), and CD supplemented with AS at 1, 5, and 10 % inclusion levels and APC at 5, 10, and 15 % inclusion levels. Diet did not affect eggshell Ca (P=0.1168) and P (P=0.8212) levels, and feed conversion ratio (P=0.136), but the 10 % FS reduced body weight gain (P=0.044), hen day egg production (P= 0.000) and feed intake (P<.0001) compared to other treatments. The yolk lightness L* was reduced (P=0.030) by 5 % APC compared to 10 % APC, redness a* was reduced (P= 0.002) by 10 % FS and 15 %APC compared to 10 %APC, CD, and 1 % AS. The 10 % FS and 15 %APC also reduced (P<0.001) yellowness *b compared to 1 %AS and 5 %APC. Apparent metabolizable energy (AME) and nitrogen-corrected apparent metabolizable energy (AME_n) increased (P<0.001) in 10 %FS and all AS and APC levels compared to CD. Compared to CD (87 %), ATTD of energy was increased (P<0.001) in hens fed 10 %FS (93 %), 1 %AS (93 %), and 15 %APC (92 %). However, 10 %FS (78.7 %) and 1 %AS (81.7 %) had higher (P=*0.011*) ATTD of P than 10 %APC (64.6 %). Similarly, ATTD of Ca was reduced (P < 0.001) in hens fed 10 %APC compared to CD and 10 %AS. Compared to other treatments, total n-3 and stearidonic acids were increased (P < 0.001) by 10 %FS and 10 %AS, respectively, and the total n-6 FAs and linoleic acid were highest (P=0.001) in 15 %APC. Both 10 %AS and 10 %FS increased (P < 0.001) eicosapentaenoic, docosahexaenoic, and alpha-linolenic acid, compared to CD. The n-6/n-3 ratio was reduced (P < 0.001) by 10 %FS and 10 %AS compared to APC and CD. Dietary treatments modulated fecal microbiota differently, but notably, *Lactobacillus* was more abundant when hens were fed 5 %AS compared to other treatments. In conclusion, the dietary supplementation of 10 %AS increased n3-FAs deposition in eggs similar to 10 % FS. However, 10 %FS reduced production performance. All levels of AS and APC increased diet metabolizable energy with no negative effect on production performance.

Introduction

Omega-3 fatty acids (n-3 FAs) are polyunsaturated fatty acids (PUFA) that cannot be produced by humans and birds, therefore they are essential fatty acids (Moran et al., 2019; El-Zenary et al., 2022). They are known for their health benefits such as being anti-inflammatory and reducing the occurrences of heart diseases (Novak and Scheideler, 2001; Moran et al., 2019; El-Zenary et al., 2022). These n-3 FAs include alpha-linolenic acid (ALA) and very long chain n-3 FA (VLCn-3 FAs) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). ALA undergoes desaturation and elongation to VLCn-3 FA (Moran et al., 2019; El-Zenary et al., 2022). EPA and DHA are beneficial to human health and have been recommended to be part of the human diet in adequate amounts. However, these recommended levels are yet to be met in various locations globally (Moran et al., 2019). Fortifying common food consumed globally with n-3 FAs has been a strategy to improve n-3 FAs intake by humans (Raza et al., 2016; Moran et al., 2019). Fatty acids are easily deposited in eggs and eggs are

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reasonably available for human consumption (Raza et al., 2016). Dietary supplementation of fatty acids has been used to enhance poultry egg n-3 FAs composition (Moran et al., 2019). A previous study demonstrated that n-3 FAs-enriched eggs increased plasma EPA and DHA levels and reduced the n-6/n-3 ratio in humans (Moran et al., 2019). ALA-rich plants are included in the diet of laying hens to enrich eggs with n-3 FA (Moran et al., 2019). ALA is a long-chain FA and part of triglycerides that are stored as energy sources in the adipocytes while the EPA and DHA are the stored forms of phospholipids deposited in the yolk.

There has been an increased demand for n-3 FA eggs (Ehr et al., 2017). The fat composition of an egg is limited to 10 % which can be saturated to its peak level depending on the dietary fat composition. The n-3 FAs deposit in the yolk depends on the dietary ALA, EPA, DHA, and total omega-3 fatty acid composition (Ehr et al., 2017). Laying hens make adjustments to dietary n-3 FAs within 2 weeks of inclusion, after which they process and deposit the n-3 FAs in the yolk (Ehr et al., 2017). Poultry are limited in modifying dietary FAs because they lack the elongase and desaturase enzymes responsible for the further metabolic action of long-chain FA (Ehr et al., 2017). Marine products such as oily fish and algae are rich in n-3 FAs; however, the amount of marine products needed to meet global human n-3 FAs needs surpasses the amount available (Novak and Scheideler, 2001). Oilseeds have been extensively studied and adopted for n-3 FAs enrichment purposes in egg yolks (Ehr et al., 2017). Eggs have been enriched with n-3 FAs using flax seed (Aziza et al., 2013; Yassein et al., 2015; Ehr et al., 2017; Panaite et al., 2020) because flaxseed has a rich ALA composition of up to 23 % and an oil composition of 34 % (Aziza et al., 2013; Goldberg et al., 2016). Ahiflower (*Buglossoides arvensis*) is a herb indigenous to northern Europe and Asia and has been planted as a weed in wheat-growing regions such as Australia and northern and southern America. It can also be cultivated in cold temperate regions such as the UK (Cumberford and Hebard, 2015). Cumberford and Hebard (2015) reported that ahiflower seed (AS) has the highest stearidonic content (18-20 % SDA) when compared to other oilseeds. SDA is produced in the body from ALA from nuts, seeds, and oil seeds. This occurs by the metabolic activity of the desaturase enzyme (Δ6D). EPA is more readily available when SDA is consumed than when ALA is consumed. The AS also has a 5-6 % gamma-linolenic acid (GLA) composition (Cumberford and Hebard, 2015) and GLA together with n-3 FAs play a positive role in suppressing inflammation in humans (Sergeant et al., 2016). The ahiflower oil is rich in SDA (up to 20 %) and ALA (up to 46 %) (El-Zenary et al., 2022). Only a single study has investigated the effects of dietary supplementation with ahiflower oil at inclusion levels of 7.5 % and 22.5 % in broilers and another study in laying hens (El-Zenary et al., 2022, 2023). The results from the broiler study showed that the breast, thigh, adipose tissue, and plasma were enriched with VLC n-3 FAs in comparison with soybean oil supplementation (El-Zenary et al., 2022). In the laying hens, the study showed that there were increased VLC n-3 FAs in the yolk (El-Zenary et al., 2023).

Furthermore, AS can serve as a source of nutrients for laying hens as it contains 12.6 % CP, 3,031 kcal/kg gross energy (GE), 13.5 % Ca, and 0.55 % P (Table 1) but its nutritional benefit has not been determined in poultry. Ahiflower oil is extracted from the seed by expeller pressing and the residual expeller press cake contains 20 % CP, 2300 kcal/kg GE, 11.5 % Ca, 0.58 % P (Table 1). The nutritional contents of AS and ahiflower press cake (APC) suggest that they could partially replace calcium, energy, and protein sources in laying hens' diets. Particularly, the Ca content of AS and APC surpasses that of any known poultry plant-based feed ingredient including soybean meal (0.37 % Ca) and wheat (0.05 % Ca) (NRC, 1998). However, the current literature has no information regarding the digestibility of Ca and P in AS and APC. The strength of eggshells remains a major concern of the egg industry because eggs with inferior shell quality are a leading source of economic losses to poultry producers (Hamilton et al., 1979). Therefore, Ca is an important ingredient in egg production and sustainable alternative sources should be sought. APC and AS could then be used to partially replace conventional Ca, energy, and protein sources in laying hens. Moreover, APC does not currently have any profitable use in the food chain and constitutes an environmental burden. Ahiflower oil is gaining more attention in human nutrition (www.ahiflower.com); thus, more press cakes will be continually produced. It is therefore imperative to find alternative uses for the press cake in the animal industry. Feed represents approximately 60 to 70 % of the total cost of poultry production (Zampiga et al., 2021). If the cost of production could be potentially reduced using a cheaper source of nutrients, this would not only benefit the egg producers, but also the consumers through a reduction in the price of eggs. Also, the residual oil in the APC can help to enrich laying hen diets with n-3 polyunsaturated fatty acids to generate value-added eggs for human consumption. If diets are formulated to closely match hens' nutritional requirements, the high fiber content and the soluble carbohydrates in the APC (23 % non-starch polysaccharides, NSP, and 22 % neutral detergent fiber, NDF) and AS (18 % NSP and 14.5 % NDF) could lead to the proliferation of beneficial bacteria in the hens' lower gut, thereby promoting gut health.

Thus far, no study has been conducted using AS or APC in poultry and no information is available on the AME_n, nutrient digestibility, and egg fatty acid deposition when AS or APC is fed to laying hens. Therefore, this study aimed to determine the effect of APC and AS as alternative calcium, energy, and protein sources on production performance, egg quality, eggshell mineral contents, and egg yolk fatty acid enrichment. We hypothesized that AS and APC will increase the n-3 FAs concentration in egg yolk, AME_n, eggshell P and Ca levels, alter fecal microbiota composition, and will not negatively affect the production performance and apparent total tract digestibility of Ca and P in laying hens.

Table 1
Fatty acid profile and nutrient composition of ahiflower seed and ahiflower press cake.

Fatty acids, mg/g	Ahiflower press cake	Ahiflower seed
Saturated fatty acids		
Stearic acid (C18:0)	1.36	2.87
Palmitic acid C16:0	3.79	8.73
Total	5.68	12.92
Monounsaturated fatty acids		
palmitoleic acid (C16:1)	0.04	0.09
Oleic acid (C18:1)	4.62	9.09
Total	5.93	10.59
Polyunsaturated fatty acids		
Stearidonic acid (SDA, C18:4 n-3)	0.36	0.86
Linoleic acid (LA, C18:2 n6)	0.98	1.54
γ-linolenic acid (GLA, C18:3 n6)	0.16	0.32
α-linolenic acid (ALA, C18:3 n3)	1.49	2.95
Arachidonic acid (AA,C20:4 n6)	0.02	0.04
Eicosapentaenoic acid (EPA, C20:5 n-3)	0.02	0.02
Docosahexaenoic acid (DHA, C22:6 n3	0	0.10
Total	3.13	5.98
Total omega fatty acids		
n-3	1.87	3.93
n-6	1.26	2.05
n-6/n-3 ratio	0.67	0.52
Total fatty acid	14.73	29.49
Nutrient composition		
Crude protein (%)	20.13	12.62
Gross energy (kcal)	2330	3031
Calcium (%)	11.48	13.51
Phosphorus (%)	0.58	0.55
Neutral detergent fiber (%)	22.30	14.53
Total dietary fiber (%)	32.18	24.01
Non-starch polysaccharide (mg/g)		
Arabinose (mg/g)	6.19	4.60
Xylose (mg/g)	2.55	-
Mannose (mg/g)	2.91	1.89
Galactose (mg/g)	5.61	4.58
Glucose (mg/g)	84.34	68.09
Uronic Acids (mg/g)	130.8	100.5
Total non-starch polysaccharide (mg/g)	232.4	179.7

Materials and methods

Birds, diets, and housing

The animal care protocol for this study was approved by Dalhousie University animal care committee (Animal Use Protocol # 20230103). A total of two hundred and forty (240) white leghorn (Lohman Lite) hens (64 weeks old) were housed at the Atlantic Poultry Research Centre, Dalhousie University (originally obtained from commercial suppliers at the pullet stage). The birds were housed in 5 2-tier movable cage systems. Five birds were randomly selected and stocked per cage compartments (500 cm^2/bird) in a room where the lighting, ventilation, and heat were controlled for the optimum performance of the birds. The birds were provided 16 h of light, 8 h of darkness, and incandescent bulbs were used as a source of light throughout the experiment. The 48 cages were replicates representing experimental units; 6 of these experimental units made up 1 treatment, and as such, the trial had 8 treatments consisting of 6 replicates each. The birds were randomly assigned to these eight experimental diets representing various treatments. The trial was conducted for 12 weeks, which was divided into 3 periods with four weeks per period; the birds were 68, 72, and 76, weeks old at the end of every period. The diets were corn-soybean meal based (isocaloric and isonitrogenous) in mash form and were formulated following the guidelines for Lohman Lite hens. As presented in Table 2, the diets included control (CD), CD + 10 % flax seed (FS), CD + 1 % ahiflower micronized seed (AS), CD + 5 %AS, CD + 10 % (AS), CD + 5 % ahiflower presscake (APC), CD + 10 % APC, CD+15 % APC. Ahiflower micronized seed and APC (supplied by Natures Crops International) were incorporated in the respective formulations to replace energy, protein and Ca yielding ingredients (Table 2) in the basal diet. In the last period of the trial, acid-insoluble ash (0.8 %) was added to the diet as an indigestible marker. Birds had access to water *ad libitum* from a nipple drinker.

Production performance

On d 0 and the end of each period (i.e., d 28, 56, and 84), individual body weight (BW) of birds and feed intake per cage were measured to determine average BW gain (BWG) per period (ABWGP, by subtracting the initial body weight from the weight of birds in period and previous BW from the current period) and average feed intake (FI) per period (AFIP). Daily, the number of eggs laid (EN) was collected to determine EN per period (ENP) and weighed to determine the average EW per period (AEWP). In addition, at the end of each period, feed conversion ratio (FCR) and hen-day egg production (HDEP) were calculated using the formulas below:

FCR = AFIP ÷ AEW

HDEP = ENP ÷ number of hens per cage.

Egg yolk fatty acid analysis

At the end of the trial, two eggs were collected from each cage randomly. Each yolk was separated from the albumen and collected into a 50ml tube per yolk representing 2 replicates per cage. The yolks were

Table 2
Ingredients and nutrient composition of experimental diets.

	Treatments[1]							
Ingredient (%)	**Control**	**10 %FS**	**1 %AS**	**5 %AS**	**10 %AS**	**5 %APC**	**10 %APC**	**15 %APC**
Corn	51.11	46.28	50.66	48.83	46.55	48.72	46.33	43.93
Soybean meal	23.79	19.77	23.57	22.69	21.59	22.48	21.17	19.85
Wheat	10	10	10	10	10	10	10	10
Flax seed	-	10	-	-	-	5	-	-
Ahiflower seed	-	-	1	5	10	-	-	-
Ahiflower press cake	-	-	-	-	-	5	10	15
Limestone	4.91	4.89	4.78	4.25	3.59	3.98	3.05	2.14
Shell mix[2]	2.46	2.45	2.39	2.13	1.8	1.99	1.52	1.04
Oyster shell	2.45	2.45	2.39	2.12	1.79	1.99	1.52	1.04
Soy oil mature	1.9	1.16	1.85	1.64	1.38	2.49	3.09	3.69
DICAL Phos 21 P	1.18	0.86	1.17	1.15	1.12	1.17	1.17	1.16
Celite	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
DL-Methionine premix[3]	0.55	0.52	0.8	0.54	0.52	0.53	0.52	0.5
Vit-Min premix[4]	0.5	0.5	0.55	0.5	0.5	0.5	0.5	0.5
Salt	0.34	0.33	0.5	0.35	0.35	0.35	0.35	0.35
Lysine HCl	100	100	100	100	100	100	100	100
Calculated Analysis								
AME_n (Kcal/kg)	2800	2800	2800	2800	2800	2800	2800	2800
Crude protein (%)	15.91	15.91	15.91	15.91	15.91	15.91	15.91	15.91
Calcium (%)	4	4	4	4	4	4	4	4
Avail. phosphorus (%)	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Sodium (%)	0.16	0.16	0.16	0.16	0.16	0.16	0.85	0.16
Lysine (%)	0.9	0.86	0.9	0.88	0.86	0.83	0.75	0.83
Methionine+ cystine (%)	0.75	0.75	0.75	0.75	0.75	0.75	0.21	0.75
Tryptophan	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
Determined Analysis[5]								
Crude protein (%)	17.6	18.65	17.85	17.35	17.2	18.8	17.85	17.9
Calcium (%)	3.13	4.49	3.349	4.63	4.82	3.62	4.06	3.45
Total phosphorus (%)	0.48	0.49	0.56	0.56	0.55	0.61	0.58	0.53
Sodium (%)	0.09	0.16	0.20	0.20	0.24	0.17	0.18	0.18
Neutral detergent fibre (%)	7.71	8.40	8.34	8.21	8.56	9.10	11.00	11.32

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.

[2] Shell mix is a commercial product manufactured from selected limestone and is screened to meet defined sizing criteria primarily for the poultry industry.

[3] Supplied per kg diet; vitamin A, 8000 IU; vitamin D_3, 2500 IU; vitamin E, 60 mg; vitamin K, 2.97 mg; 7.6 mg; DL Ca-pantothenate, 7.2 mg; vitamin B_{12}, 0.012 mg; niacin, 30.7 mg; folic acid, 0.66 mg; choline chloride, 641 mg; biotin, 0.16 mg; pyridoxine, 4.0 mg; thiamine, 1.9 mg; manganous oxide, 70.2 mg; zinc oxide, 80 mg; copper sulphate, 25 mg; selenium 0.15 mg; ethoxyquin, 50 mg; corn, 2572 mg; ground limestone, 500 mg.

[4] Supplied per Kg premix. DL-Methionine 0.5kg; corn 0.5Kg.

[5] Diet samples were analysed in duplicate

analyzed in duplicate to determine the FA profile at the University of Guelph lipid analytical laboratories following the procedure described by Aguillón-Páez et al. (2020).

Determination of nutrient digestibility

Fresh excreta samples (free of feathers and feed particles) were collected from each cage three times/day on the last two days of the trial at 3-h intervals into aluminum pans. The samples were freeze-dried and ground using a coffee grinder, after which they were stored at room temperature and used for analyzing acid insoluble ash (AIA), dry matter (DM), gross energy (GE), CP (N × 6.25), and minerals (Ca and P).

Apparent metabolizable energy corrected for nitrogen was calculated using the formula described by Leeson and Summers (2001).

Feed and diet samples were collected and analyzed for DM, GE, CP, Ca, and total P.

Chemical analysis

The AIA in the diet and fecal samples was determined in triplicate as described by Vogtmann et al. (1975). Dry matter was determined following the AOAC (1990) method for analysis. The GE of feed and diet was determined in duplicate using the bomb calorimeter, standardized with benzoic acid (Model 1281, Parr Instruments, Moline, IL, USA). Leco Protein analyzer (Model Leco–FP–528L, Leco Corporation, St. Joseph, MA, USA) was used for analyzing CP content in feed and fecal samples. Ca and P levels in diet, feces, and eggshells were determined using ICP-MS (Thermo Fisher Scientific, Bremen, Germany) following standard laboratory procedures. The non-starch polysaccharides and total dietary fiber contents of AS and APC were determined by the component analysis method as previously reported by Adewole et al. (2016).

The digestibility for various components was calculated using the formula:

Apparent total tract digestibility (ATTD) of nutrient (CP, Ca, P), % = 1− (% AIA $_{feed}$ / % AIA $_{feces}$) × (% nutrient $_{feces}$ /% nutrient $_{feed}$) (Aziza et al., 2013).

Where % AIA $_{feed}$= percent AIA in the feed

% AIA $_{feces}$ = percent AIA in the feces

% nutrient $_{feces}$ = percent nutrient in the feces

% nutrient $_{feed}$ = percent nutrient in the feed

Egg quality

At the end of each period (weeks 4, 8, and 12), 4 eggs were collected per cage for egg quality measurements including egg weight, egg breaking strength, specific gravity, shell weight, shell thickness, yolk color, albumen height, and Haugh unit. The specific gravity was determined by dipping eggs in salt solutions with densities that varied from 1.066 to 1.098 and graded at an increasing interval of 0.004. The density was recorded as the value for specific gravity as eggs floated to the water's surface. The eggshell-breaking strength was determined using a TA.XT plus texture analyzer (Texture Technologies Corp., Scarsdale, New York, USA) with version 2.0.7.0 Exponent Stable Micro Systems software, with the wide end of the egg kept in support vertically and a 30 kg solid used to apply force using a probe. The albumen height was determined using a micrometer. The yolk was separated from the albumen and used to determine the yolk color using a colorimeter by calibrating a transparent bowl wherein each yolk was collected and each yolk color was determined using the color system of CIE (Commission Internationale de L'Eclairage) to determine the yolk color which involves the use of L* to indicate the level of lightness measured from 0-100, representing darkness to lightness, a* to show how reddish the egg is, indicating the level of red-green color shade, with the more elevated positive value representing a redder shade and the b* (yellowness) showing the level of yellow-blue color, wherein a more elevated positive value is yellowish (Grčević et al., 2019; Yüceer and Asik, 2020). The correlation between the egg weight and albumen height was used for Haugh unit determination using the formula of Haugh (1937) as follows:

HU = 100 × log (h – 1.7W0.37 + 7.6)

H = albumen height (mm)

W = egg weight (g)

Eggshells were washed under running water to remove any albumen and yolk, dried for 5 days, followed by the determination of shell thickness and weight. The shell thickness was measured using a lobster texture analyzer; the shells were pooled per cage and then ground into a fine powder and stored to determine eggshell calcium and phosphorus levels.

DNA extraction, 16S rRNA gene sequencing, and data analysis

DNA was extracted from the fecal samples using QIAamp® Stool Kit using the manufacturer's instructions. Thermo nanodrop2000 UV microscope spectrometer was used to quantify overall DNA. The different bacterial communities in the feces were determined using the Illumina protocol to sequence the V3-V4 hypervariable region of the 16srRNA gene on Illumina MiSeq at the integrated microbiome resource, Dalhousie University (Halifax, Nova Scotia). The analysis was carried out using the microbiome helper pipeline (https://github.com/LangilleLab/microbiome_helper/wiki, accessed on 30 October 2023) and the sequence reading was through QIME2 using the amplicon sequence variants (ASVs) created by Deblur. The individual alpha diversity of the different treatments was viewed using the rarefaction curves and compared between treatment groups using Shannon boxplots and the Kruskal-Wallis statistical test fixed at $P < 0.05$. Also, UniFrac PCoA plots were used to examine the beta diversity between the treatment's taxonomic classification. Furthermore, Stacked bar charts were used to view the relative abundance of distinct taxonomic classifications. STAMP was used to examine the relative abundance of microbial communities among treatments. To view the percentage composition of the microbiota, the STAMP application (http://kiwi.cs.dal.ca/Software/STAMP) was used. The statistical test was ANOVA ($P< 0.05$) with a post-hoc test using Tukey-Kramer.

Statistical analysis

All data collected were analyzed as a completely randomized design using the mixed procedure of SAS v9.4 (Statistical Analysis Software Program). The Turkey test was used to compare the resultant mean values and tabulated as means and standard error of the means (SEM). A probability value less than 0.05 ($P<0.05$) was identified as significant. Also, results for AME_n and production performance were log (+1) transformed for normalization of data distribution.

Results

Ahiflower seed, press cake, and diet fatty acid profile

Table 1 and Table 3 show the FAs profiles of AS and APC, and the dietary treatments, respectively. The AS had 0.86 % SDA, 1.54 % LA, GLA of 0.32 %, 2.95 % ALA, 3.93 % total n-3, 2.05 % total n-6, and a total fatty acid content of 29.49 % while the APC had 0.36 % SDA, 0.98 % LA, 0.16 % GLA, 1.49 % ALA, 1.87 % total n-3, 1.26 % total n-6, and total fatty acid content of 14.73 %. The AS had a higher GE (3,031 kcal/kg) and Ca (13.51 %) than APC which had a GE of 2,330 kcal/kg and 11.48 % Ca. The dietary FA profiles showed that SDA (2.30 mg/g) and GLA (1.03 mg/g) were highest in the diet supplemented with 10 %AS compared to other treatments. The 15 % APC had the highest LA (28.41mg/g) and total n-6 (28.67 mg/g). The 10 % FS-supplemented diet had the highest ALA (20.26 mg/g), PUFA (41.77 mg/g), total n-3 (20.37 mg/g), and total FAs (62.72 mg/g). Also, 10 % FS had the lowest n-6: n-3 (1.05) compared to other treatments as shown in Table 2.

Table 3
Fatty acid profile of dietary treatment.

	Treatments[1]							
Fatty acids (%)	**Control**	**10 %FS**	**1 %AS**	**5 %AS**	**10 %AS**	**5 %APC**	**10 %APC**	**15 %APC**
Saturated fatty acids								
Stearic acid (C18:0)	1.36	1.84	1.25	1.34	1.16	1.41	1.86	1.91
Palmitic acid C16:0	5.44	5.64	4.89	5.13	4.75	5.16	6.43	6.57
Total	7.33	8.02	6.61	6.95	6.33	7.08	8.91	9.15
Monounsaturated fatty acids								
Palmitoleic acid (C16:1)	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06
Oleic acid (C18:1)	10.00	12.73	8.54	8.90	8.46	8.77	11.05	11.67
Total	10.21	12.93	8.74	9.20	8.82	8.97	11.29	11.94
Polyunsaturated fatty acids								
Stearidonic acid (SDA, C18:4 n-3)	0	0	0.46	2.30	3.61	0.03	0.04	0.04
Linoleic acid (LA, C18:2 n6)	23.93	21.08	20.84	20.70	18.00	22.21	27.53	28.41
γ-linolenic acid (GLA, C18:3 n6)	0.01	0.01	0.14	0.67	1.03	0.02	0.02	0.04
α-linolenic acid (ALA, C18:3 n3)	2.46	20.26	3.14	7.44	9.91	2.46	3.25	3.32
Arachidonic acid (AA,C20:4 n6)	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02
Eicosapentaenoic acid (EPA, C20:5 n-3)	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.01
Docosahexaenoic acid (DHA, C22:6 n3	0	0	0	0.009	0	0	0	0
Total	26.62	41.77	24.77	31.35	32.79	24.94	31.06	32.04
Total omega fatty acids								
n-3	2.47	20.37	3.61	9.77	13.53	2.50	3.31	3.37
n-6	24.15	21.40	21.15	21.58	19.26	22.43	27.76	28.67
n-6/n-3 ratio	9.76	1.05	5.85	2.21	1.42	8.96	8.40	8.50
Total fatty acids	44.16	62.72	40.11	47.50	47.94	40.98	51.27	53.13

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.

Production performance

As presented in Table 4, FI was significantly reduced ($P<.0001$) by 10 % FS compared to CD, 10 %AS, and all inclusion levels of APC. HDEP was significantly reduced ($P=0.000$) from dietary supplementation of 10 %FS compared to all AS and APC inclusion levels. Overall, 10 %FS reduced BWG ($P=0.044$) compared to 15 % APC. In contrast, dietary treatment had no significant effect on FCR ($P=0.136$). There was a significant difference in treatment-period interaction on FCR, FI, and HDEP, such that in period 1 only, the 10 %FS significantly reduced ($P=0.002$) HDEP compared to CD, 1, 5, and 10 % AS and 10 % APC. A significant effect ($P<.0001$) of period on the production performance was observed (Table 4). The FI and HDEP increased, whereas the FCR and BWG significantly decreased, as hens increased in age. Overall Period effect was observed on production performance, but no significant difference between the FCR in period 1 and period 2 was observed. BWG differed significantly ($P<.0001$) between period 1 and 2, while FI and HDEP differed significantly ($P=0.001$) between periods 1 and 3. Furthermore, a mortality rate of less than 5 % was observed throughout the trial.

Table 4
Production performance of laying hens (64wk-76wk) fed diets supplemented with flax seed, ahiflower seed, and ahiflower press cake.

	Treatments[1]										P-value		
Parameters	Control	10 % FS	1 % AS	5 % AS	10 % AS	5 % APC	10 % APC	15 % APC	Period	SEM[2]	Treatment	Period	Period* Treatment
Feed conversion ratio													
Period 1	1.8	1.8	1.9	1.8	1.8	1.9	1.8	1.96	1.85^{y}	0.05	0.251	0.004	0.0020
Period 2	1.7	1.7	1.8	1.8	1.8	1.8	1.8	1.9	1.84^{y}	0.05	0.177		
Period 3	1.8	1.9	1.7	1.8	1.9	1.8	1.9	1.9	1.78^{x}	0.06	0.214		
Overall	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.9		0.03	0.000		
[3]Body weight gain (g/bird)													
Period 1	-70	-128	-63	-91	-74	-76	-60	-49	-76.32^{x}	16.23	0.066	<.0001	0.6621
Period 2	-25	-41	-25	-7	-37	-6	-9	4	-18.28^{y}	11.86	0.102		
Period 3	-70	-128	-63	-91	-74	-76	-61	-49	-21.08^{y}	16.22	0.066		
Overall	-38^{ab}	-69^{b}	-34^{ab}	-36^{ab}	-43^{ab}	-36^{ab}	-29^{ab}	-24^{a}		8.86	0.044		
Feed intake (g/bird/d)													
Period 1	110^{a}	99^{b}	107^{a}	108^{a}	109^{a}	106^{ab}	109^{a}	110^{a}	107^{x}	1.6	0.000	0.001	0.0062
Period 2	108^{ab}	98^{c}	105^{bc}	107^{ab}	109^{ab}	110^{ab}	113^{ab}	115^{a}	108^{xy}	2.1	<.0001		
Period 3	114	105	106	110	112	111	112	110	110^{y}	2.1	0.290		
Overall	111^{a}	102^{b}	105^{ab}	108^{ab}	110^{a}	109^{a}	111^{a}	112^{a}		1.4	<.0001		
Hen-day egg production (%)													
Period 1	90.7^{a}	79.0^{b}	90.1^{a}	91.1^{a}	94.5^{a}	88.9^{ab}	90.7^{a}	86.6^{ab}	89.5^{y}	2.24	0.002	0.001	0.0100
Period 2	91.3	89.8	93.0	92.3	93.8	95.8	91.6	93.8	92.7^{x}	1.97	0.530		
Period 3	90.1	90.7	91.7	94.8	89.9	95.1	94.1	97.3	92.9^{x}	2.40	0.147		
Overall	90.1^{ab}	86.5^{b}	92.7^{a}	92.7^{a}	93.2^{a}	94.2^{a}	92.7^{a}	93.3^{a}		1.18	0.000		

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.

[2] SEM = Standard error of the mean. Each period is 4 weeks. Superscripts a-c connote significant differences in the same row ($P < 0.05$) while superscripts x-y connote significant differences in the same column ($P < 0.05$). Values are expressed as mean ± standard error (SE).

[3] Body weight gain was obtained by subtracting previous body weight from current body weight, and for period 1, the initial body weight was subtracted from the body weight recorded for that period.

Nutrient digestibility

The nutrient digestibility values are shown in Table 5. The AME_n and AME were significantly different across dietary treatments with the highest ($P<0.001$) in hens fed 10 % FS compared to CD, but this did not differ from hens fed diets supplemented with 1 %AS, 10 %AS, and 15 % APC. ATTD of energy was significantly reduced ($P=0.011$) by 10 %APC compared to 10 %FS, 1 %AS, 10 %AS, and 15 % APC dietary supplementation which did not differ from CD, 5 % AS, and 5 % APC. The ATTD of P was significantly reduced ($P=0.011$) in hens fed 10 %APC compared to hens fed 1 %AS, 10 %AS, and 15 %APC but did not differ from CD,5 %AS, and 5 %APC. The ATTD of Ca was lowest ($P<0.001$) in hens fed 10 %APC compared to hens fed 10 %AS.

Fatty acid profile of egg yolk

Dietary treatment had no significant effect on egg yolk saturated fatty acid (FA) and monounsaturated fatty acid (MUFA) compositions (Table 6). The highest total n-3FA deposits in egg yolk were observed from hens fed 10 % FS which increased by 76 % ($P<0.05$) compared to the CD. The SDA deposit was highest ($P<.0001$) in egg yolks of hens fed 10 %AS which increased by more than 8 times compared to egg yolks from hens fed CD. EPA deposit in egg yolks was higher ($P<.0001$) when birds were fed 10 %AS and 10 %FS supplemented diet compared to the CD. ALA was highest ($P<.0001$) in eggs from hens fed 10 % AS and 10 % FS. Meanwhile, DHA deposition was highest ($P<.0001$) in egg yolks from hens fed 5 and 10 % AS, and 10 %FS compared to CD. The LA deposit in egg yolks was reduced ($P=0.0023$) in hens fed 10 % FS compared to 15 % APC. Hens fed 15 %APC supplemented diet increased ($P=0.001$) total n-6 FA in egg yolks compared to eggs from hens fed CD, 10 %FS, 1 %AS, and 10 %AS but did not differ from hens fed diet supplemented with 5 %AS, and 5 and 10 %APC. Egg yolk from birds fed 10 %FS had the lowest ($P<.0001$) GLA deposit compared to other treatments. Furthermore, egg yolk from birds fed 10 %FS and 10 %AS had the lowest ($P<.0001$) arachidonic acid (AA) deposit compared to eggs from hens fed diet supplemented with CD, 1 %AS, and all press cake inclusion levels but did not differ from 5 % AS. Total PUFAs was highest ($P=0.028$) in egg yolks from hens fed 15 % APC compared to eggs from hens fed 1 %AS but did not differ from other treatments.

Egg quality and eggshell calcium and phosphorus levels

The egg quality parameters determined were egg weight, egg breaking strength, specific gravity, shell weight, shell thickness, yolk color, albumen height, and Haugh unit. Overall, dietary treatment did not affect ($P>0.05$) the eggshell Ca and P levels and the following egg quality parameters - egg weight, egg breaking strength, specific gravity, shell weight, shell thickness, yolk color, albumen height, and Haugh unit. The yolk color (L*, a*, b*) was significantly influenced by the diets with the L* score of eggs from hens fed 5 %APC diet significantly lower ($P<.0001$) than in eggs from hens fed 10 %APC but did not differ significantly from other treatments. The a* color score in eggs from hens fed 10 %FS and 15 %APC was significantly reduced ($P=0.002$) compared to CD and 1 % AS but did not differ significantly from other treatments. The b* color score was significantly reduced ($P<.0001$) in eggs from hens fed 10 %FS and 15 %APC than in eggs from hens fed 1 % AS and 5 %APC but did not differ from the other treatments (Tables 7–9).

Fecal microbiota richness and diversity

We used the Shannon index to determine the Alpha diversity, to account for the richness and even diversity of the microbiota community. From our observation, treatment had no significant effect ($P>0.5$) on alpha diversity measured by the Shannon index (Fig. 1). Furthermore, Beta diversity was determined using Unifrac PCOA plots. It showed that the microbial composition was similar across all treatments, being sparsely populated (Fig. 2).

Taxonomical classification of fecal microbiota

The relative abundance of the microbial communities at different taxonomic classifications was evaluated across all treatments. In this study, we observed 9 phyla and 140 genera across fecal samples. The most abundant phylum across all treatments were Firmicutes (86.4 %), and Bacteroidata (9.8 %). At the genus level (Fig. 3), *Lactobacillus, Romboutsia, Faecalibacterium, Bacteroides, Ruminococcus (torques_group), Clostridium_sensu_stricto_1, Subdoligranulum, Blautia, Alistipes* were the most abundant genera. Although *Lactobacillus* was the most dominant across all treatments, it was more abundant when hens were fed 5 % AS (42.93 %; Fig. 3). Our stamp analysis (Fig. 4) showed that dietary supplementation of 15 % APC significantly increased *Merdibacter* compared to 1 % and 5 % AS. We also observed that the relative abundance of *Erysipeiotrichaceae_unclassified* was significantly reduced among hens fed 10 % FS diet compared to CD. Furthermore, *Butyricicoccus, Coprobacter*, and *Erysipelatoclostridium* were significantly lower by 1 % AS than CD. The abundance of *Bacteroides* and *Erysipelatoclostridium* were significantly lower in 5 %AS than in CD. 10 % AS significantly reduced *Butyricicoccus, Coprobacter, Erysipelatoclostridium, eubacterium, and Negativibacilus* in feces compared to CD. A higher abundance of *Christensenellaceae R-7_group* was observed in hens fed 5 % APC diet compared to CD. We observed that dietary supplementation of 10 % APC significantly reduced *Coprobacter, Bacteroides* abundance compared to CD. In addition, *negativibacilus* abundance was significantly higher in 15 % APC diet than in CD, while the abundance of *butyricicoccus, coprobacter, family_XIII_UCG-001, faecalitalea* was significantly reduced by 15 % APC diet than CD.

Table 5
Nitrogen corrected apparent metabolizable energy (AMEn) and nutrient digestibility of laying hens fed supplemented with flaxseed, ahiflower seed, and ahiflower press cake.

	Treatments[1]									
Parameters	**Control**	**10 % FS**	**1 % AS**	**5 % AS**	**10 % AS**	**5 % APC**	**10 % APC**	**15 % APC**	**SEM[2]**	***P*-value**
AME (kcal/kg)	2825^{e}	3388^{a}	3301^{ab}	3135^{cd}	3197^{bc}	3040^{d}	3047^{d}	3302^{ab}	32.95	<.0001
AME_n (kcal/kg)	2819^{e}	3378^{a}	3288^{ab}	3127^{cd}	3188^{bc}	3032^{d}	3038^{d}	3292^{ab}	32.15	<.0001
ATTD[3] of energy (%)	87.1^{bc}	92.5^{a}	92.9^{a}	89.9^{abc}	91.2^{ab}	87.5^{bc}	86.4^{c}	91.8^{a}	0.93	<.0001
ATTD of P (%)	71.6^{abc}	78.7^{ab}	81.7^{a}	72.1^{abc}	81.5^{a}	67.5^{bc}	64.6^{c}	79.0^{a}	2.56	0.011
ATTD of Ca (%)	79.5^{b}	85.9^{ab}	86.4^{ab}	80.5^{ab}	86.7^{a}	80.7^{ab}	70.2^{c}	81.4^{ab}	1.55	<.0001

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.
[2] SEM = Standard error of the mean.
[3] ATTD-Apparent total tract digestibility. Superscripts a-e connote significant differences in the same row ($P < 0.05$). Values are expressed as mean ± standard error (SE).

Table 6
Fatty acid composition of egg yolk from hens fed diet supplemented with flaxseed, ahiflower seed, and ahiflower press cake.

	Treatments[1]									
Fatty acids, mg/g yolk	Control	10 %FS	1 %AS	5 %AS	10 %AS	5 %APC	10 %APC	15 %APC	SEM[2]	*P*-value
Saturated fatty acids										
Stearic acid (C18:0)	12.2	12.3	12.3	13.3	13.3	12.8	11.9	12.7	0.01	0.645
Palmitic acid (C16:0)	33.6	30.9	34.8	34.9	34.0	34.7	31.8	34.1	1.56	0.497
Total	46.5	43.7	47.8	48.9	48.0	48.2	44.3	47.4	2.09	0.594
Monounsaturated fatty acids										
Palmitoleic acid (C16:1)	3.49	3.33	3.79	3.43	3.34	3.23	2.84	2.83	0.25	0.136
Oleic acid (C18:1)	49.2	47.4	50.0	49.7	47.8	49.5	45.0	48.5	2.17	0.763
Total	53.2	51.2	54.3	53.7	51.6	53.3	48.3	51.9	2.25	0.663
Polyunsaturated fatty acids										
Stearidonic acid (SDA, C18:4 n-3)	0.02^{c}	0.10^{b}	0.05^{c}	0.12^{b}	0.19^{a}	0.03^{c}	0.047^{c}	0.04^{c}	0.01	<.0001
Linoleic acid (LA, C18:2 n6)	19.1^{b}	18.1^{b}	18.6^{b}	20.4^{ab}	19.1^{b}	21.7^{ab}	22.0^{ab}	24.1^{a}	1.16	0.0023
γ-linolenic acid (GLA, C18:3 n6)	0.21^{a}	0.14^{b}	0.21^{a}	0.23^{a}	0.27^{a}	0.21^{a}	0.21^{a}	0.22^{a}	0.01	<.0001
α-linolenic acid (ALA, C18:3 n3)	0.84^{d}	6.71^{a}	1.03^{d}	2.83^{c}	4.56^{a}	1.03^{d}	1.13^{d}	1.29^{d}	0.22	<.0001
Arachidonic acid (AA,C20:4 n6)	2.21^{a}	1.25^{c}	2.08^{ab}	1.67^{bc}	1.37^{c}	2.27^{a}	2.24^{a}	2.37^{a}	0.10	<.0001
Eicosapentaenoic acid (EPA, C20:5 n-3)	0.03^{d}	0.19^{ab}	0.05^{cd}	0.13^{bc}	0.27^{a}	0.03^{d}	0.03^{d}	0.03^{d}	0.02	<.0001
Docosahexaenoic acid (DHA, C22:6 n3)	1.25^{b}	2.04^{a}	1.58^{b}	2.24^{a}	2.23^{a}	1.35^{b}	1.32^{b}	1.53^{b}	0.10	<.0001
Total	27.75^{ab}	29.48^{ab}	24.61^{b}	28.74^{ab}	29.13^{ab}	27.75^{ab}	28.03^{ab}	31.72^{a}	1.49	0.028
Total omega fatty acids										
n-3	2.31^{d}	9.51^{a}	2.92^{d}	5.71^{c}	7.78^{b}	2.60^{d}	2.67^{d}	3.09^{d}	0.30	<.0001
n-6	22.55^{b}	19.98^{b}	21.69^{b}	23.03^{ab}	21.35^{b}	25.16^{ab}	25.36^{ab}	28.64^{a}	1.28	0.001
n-6/n-3 ratio	9.80^{a}	2.11^{d}	7.42^{b}	4.03^{c}	2.78^{d}	9.82^{a}	9.53^{a}	9.33^{a}	0.23	<.0001
Total Fatty Acids	124.5	124.4	126.8	131.3	128.7	129.2	120.7	130.9	5.45	0.864

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.
[2] SEM = standard error of the mean. Superscripts a-d connote significant differences in the same row ($P < 0.05$). Values are expressed as mean ± standard error (SE). 6 replicates per treatment.

Table 7
External egg quality parameters of laying hens fed diet supplemented with flaxseed, ahiflower seed, and ahiflower press cake.

	Treatments[1]									***P*-value**		
Parameters	**Control**	**10 % FS**	**1 % AS**	**5 % AS**	**10 % AS**	**5 % APC**	**10 % APC**	**15 % APC**	**SEM**[2]	**Treatment**	**Period**	**Period* Treatment**
Egg weight (g)												
Initial	68.3	64.5	67.0	68.2	66.8	65.6	68.3	65.1	1.95	0.748	0.642	0.493
Period 1	65.6	63.8	64.2	65.3	66.1	64.3	66.5	65.1	1.09	0.624		
Period 2	66.4	64.9	65.5	64.7	66.7	65.5	67.5	65.6	1.02	0.525		
Period 3	65.2	65.0	64.5	64.8	65.2	63.9	64.3	65.1	1.19	0.993		
Overall	65.9	64.1	64.7	65.2	65.7	65.0	66.1	64.8	1.45	0.546		
Egg breaking strength (N)												
Initial	5156	6102	5669	5134	5581	4774	5107	4876	464.8	0.497	<.0001	0.068
Period 1	4795	5409	5192	5530	5352	5642	5236	4809	199.9	0.038		
Period 2	4778^{ab}	4087^{c}	4898^{abc}	4561^{abc}	5143^{abc}	4699^{abc}	4645^{abc}	5212^{bc}	227.0	0.035		
Period 3	4413	4255	4151	4990	4429	4777	4713	4161	307.6	0.426		
Overall	4785	4565	4797	4922	4990	5098	4705	4758	145.76	0.251		
Specific gravity (g/cm^3)												
Initial	1.08	1.09	1.09	1.08	1.08	1.08	1.09	1.09	0.00	0.538	0.756	0.003
Period 1	1.08	1.08	1.08	1.08	1.08	1.09	1.08	1.08	0.00	0.258		
Period 2	1.08	1.08	1.08	1.08	1.09	1.08	1.08	1.08	0.00	0.108		
Period 3	1.08	1.08	1.08	1.08	1.08	1.09	1.08	1.08	0.00	0.037		
Overall	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	0.00	0.518		
Shell weight (g)												
Initial	4.6	4.9	4.4	4.8	4.58	4.47	4.80	4.38	0.20	0.488	0.468	0.494
Period 1	6.2	6.4	6.3	6.42	6.36	6.42	6.54	6.25	0.11	0.373		
Period 2	6.00	6.0	6.2	6.03	6.26	6.19	6.22	6.18	0.11	0.381		
Period 3	6.11	5.99	5.92	6.24	6.11	6.19	6.16	6.21	0.13	0.601		
Overall	6.1	6.1	10.4	6.2	6.2	6.2	6.3	6.1	1.45	0.270		
Shell thickness top (mm)												
Initial	0.49	0.50	0.47	0.47	0.46	0.48	0.45	0.46	0.49	0.244	0.000	0.781
Period 1	0.61	0.55	0.59	0.59	0.54	0.62	0.56	0.65	0.05	0.808		
Period 2	0.47	0.50	0.48	0.47	0.48	0.46	0.47	0.47	0.01	0.822		
Period 3	0.44	0.45	0.41	0.42	0.45	0.53	0.42	0.50	0.03	0.104		
Overall	0.5	0.5	0.5	0.5	0.5	0.5	0.9	0.5	0.15	0.477		
Shell thickness bottom (mm)												
Initial	0.48	0.48	0.56	0.48	0.46	0.46	0.50	0.48	0.03	0.507	0.157	0.541
Period 1	0.58	0.51	0.51	0.52	0.51	0.52	0.51	0.59	0.01	0.293		
Period 2	0.47	0.48	0.49	0.48	0.51	0.48	0.52	0.49	0.01	0.078		
Period 3	0.45	0.45	0.45	0.45	0.46	0.47	0.49	0.44	0.02	0.332		
Overall	0.5	0.5	3.9	0.5	0.5	0.5	0.9	0.5	1.23	0.601		

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.
[2] [2]SEM = Standard error of the mean. Each period is 4 weeks. Superscripts a-c connote significant differences in the same row ($P < 0.05$). Values are expressed as mean ± standard error (SE)

Table 8
Internal egg quality of laying hens fed diet supplemented with flaxseed, ahiflower seed and ahiflower press cake.

Parameters	Treatments[1]								SEM[2]	*P*-value		
	Control	10 % FS	1 % AS	5 % AS	10 % AS	5 % APC	10 % APC	15 % APC		Treatment	Period	Period* Treatment
Yolk Color												
L* (Lightness)												
Initial	64.6	63.9	62.3	64.4	63.4	61.2	62.3	63.0	0.78	0.045	<.0001	0.9617
Period 1	61.5	62.4	61.8	61.8	61.9	61.2	62.3	62.2	0.28	0.082		
Period 2	61.9	61.9	61.5	61.9	61.9	61.1	62.3	61.9	0.34	0.357		
Period 3	63.6	62.9	63.4	63.5	63.3	63.1	63.1	63.3	0.33	0.798		
Overall	62.1^{ab}	62.5^{ab}	62.1^{ab}	62.2^{ab}	62.3^{ab}	61.8^{b}	62.7^{a}	62.6^{ab}	0.18	0.030		
a * (Red)												
Initial	10.5	10.9	10.8	11.2	9.7	11.1	11.5	11.3	0.63	0.492	0.002	0.157
Period 1	10.3^{ab}	9.3^{c}	9.9^{abc}	10.2^{abc}	9.7^{abc}	10.5^{a}	9.8^{abc}	9.5^{bc}	0.22	0.005		
Period 2	9.5^{ab}	8.8^{b}	10.5^{a}	9.5^{ab}	9.4^{ab}	9.5^{ab}	9.5^{ab}	9.3^{ab}	0.32	0.043		
Period 3	9.3	10.1	9.3	9.1	9.7	9.3	9.7	9.5	0.38	0.676		
Overall	10.0^{a}	9.1^{b}	10.0^{a}	9.8^{ab}	9.3^{ab}	9.9^{ab}	9.6^{ab}	9.2^{b}	0.18	0.002		
b * (Yellow)												
Initial	69.5	74.1	74.6	72.9	70.5	75.2	75.9	73.1	1.78	0.165	<.0001	0.1793
Period 1		67.8^{ab}	67.8^{ab}	67.6^{ab}	67.3^{ab}	71.0^{a}	67.3^{ab}	68.6^{ab}	0.99	0.090		
Period 2	66.3^{ab}	65.0^{b}	70.0^{a}	66.1^{b}	67.1^{ab}	68.0^{ab}	67.3^{ab}	67.6^{ab}	0.86	0.011		
Period 3	68.6	70.6	69.3	68.5	70.6	69.6	70.7	69.5	1.17	0.780		
Overall	68.7^{abc}	67.1^{c}	69.3^{ab}	67.9^{bc}	67.4^{bc}	70.3^{a}	68.5^{abc}	67.1^{c}	0.46	<.0001		
Albumen height (mm)												
Initial	7.0	5.5	5.4	5.8	5.9	5.4	6.0	6.2	0.50	0.369	<.0001	0.152
Period 1	6.3^{ab}	6.5^{ab}	6.0^{b}	6.5^{ab}	6.5^{ab}	6.7^{ab}	7.1^{a}	6.2^{ab}	0.23	0.084		
Period 2	6.6	6.6	7.3	7.3	6.9	6.4	6.5	7.0	0.38	0.566		
Period 3	6.2	5.7	6.0	5.9	6.0	6.2	5.9	6.2	0.23	0.717		
Overall	6.2	6.4	6.3	6.5	6.5	6.4	6.6	6.4	0.20	0.918		
Haugh unit												
Initial	74.1	72.2	66.1	70.3	76.9	67.7	74.6	77.8	4.24	0.463	0.614	0.552
Period 1	77.9	75.8	77.8	77.8	75.1	80.4	80.4	78.0	1.67	0.286		
Period 2	76.9	78.1	78.3	80.4	79.4	80.7	79.3	80.3	2.66	0.969		
Period 3	74.3	74.4	76.4	77.0	72.6	73.9	74.0	75.3	1.81	0.734		
Overall	76.0	76.4	71.7	78.0	76.4	79.8	77.1	78.2	1.94	0.614		

[1] FS: flax seed; AS: ahiflower micronized seed; APC: ahiflower press cake.
[2] [2]SEM = standard error of the mean. Superscripts a–c connotes significant differences in the same row ($P < 0.05$). Values are expressed as the mean standard error (SE). L* = level of lightness measured from 0-100, representing darkness to lightness. a*= shows how reddish the egg is, indicating the level of red-green color shade, with the more elevated positive value representing a redder shade. The value b* (yellowness) is the level of yellow-blue color, wherein a more elevated positive value is yellowish.

Table 9
Eggshell phosphorus and calcium level of laying hens fed diet supplemented with flaxseed, ahiflower seed, and ahiflower press cake.

Parameters	Treatments[1]								SEM[2]	*P*-value
	Control	10 %FS	1 %AS	5 %AS	10 %AS	5 %APC	10 %APC	15 %APC		
P (%)	0.09	0.10	0.09	0.09	0.09	0.09	0.09	0.08	0.004	0.1168
Ca (%)	34.66	35.04	34.91	34.25	34.47	34.58	34.52	34.59	0.35	0.8212

[1] FS - Flax seed, AS - ahiflower micronized seed, APC - ahiflower press cake.
[2] [2]SEM = Standard error of the mean. Values are expressed as mean ± standard error (SE).

Discussion

The diet nutrient composition and fatty acid profiles differed due to the varying inclusion levels of AS and APC. The diets were formulated to be isonitrogenous (15.91 % CP) but the determined CP after analysis showed that the CP content differed across the various experimental diets (17.2-18.8 %), possibly due to variations in the CP contents of ingredient batches at the feed mill. Dietary supplementation of 10 %FS was used as a reference because it is commonly used in commercial egg production as a source of n-3 FAs. Several studies have used flax seed for n-3 egg enrichment purposes (Al-Nasser et al., 2011; Aziza et al., 2013; Huang et al., 2018a). It has been reported that the antinutritional factors in flax seed such as high fiber concentration, cyanogenic glycosides, and phytic acid negatively affect nutrient utilization that could cause a reduction in AME and AME_n (Bernacchia et al., 2014; Russo and Reggiani, 2017). On the contrary, in the current study, AME and AME_n were significantly increased in hens fed 10 %FS. However, despite this, the hens on the 10 %FS showed significant reduction in production performance, arising from reduced feed intake possibly due to the presence of antinutritional factors. Flaxseed has mucilage and is attributed to have increased digesta viscosity thereby interfering with enzymes negatively, resulting in reduced nutrient digestibility and absorption (Huang et al., 2018a). All levels of AS and APC had higher AME and AME_n, compared to CD. The increased AME and AME_n in the oilseed supplemented diets could result from higher fat content, reduced feed passage rate, and increased enzyme activity in those diets due to oilseed supplementation. It is well known that dietary fat is a significant energy source. Dietary fat has also been reported to reduce the rate of food passage through the digestive tract allowing increased enzyme activities on the feed consumed and leading to more nutrient digestibility and absorption (Mateos and Sell, 1981). Huang et al. (2018a) reported that dietary supplementation of flax seed at 7.5 % and 15 % for a 58-weeks-old laying hen did not affect AME_n while Aziza et al. (2013) reported that dietary supplementation of flaxseed at 10 % reduced AME_n in 24 weeks old laying hens. Similarly, Ortiz et al. (2001) and Rodríguez et al. (2001) used day-old broilers and reported a reduction in AME_n

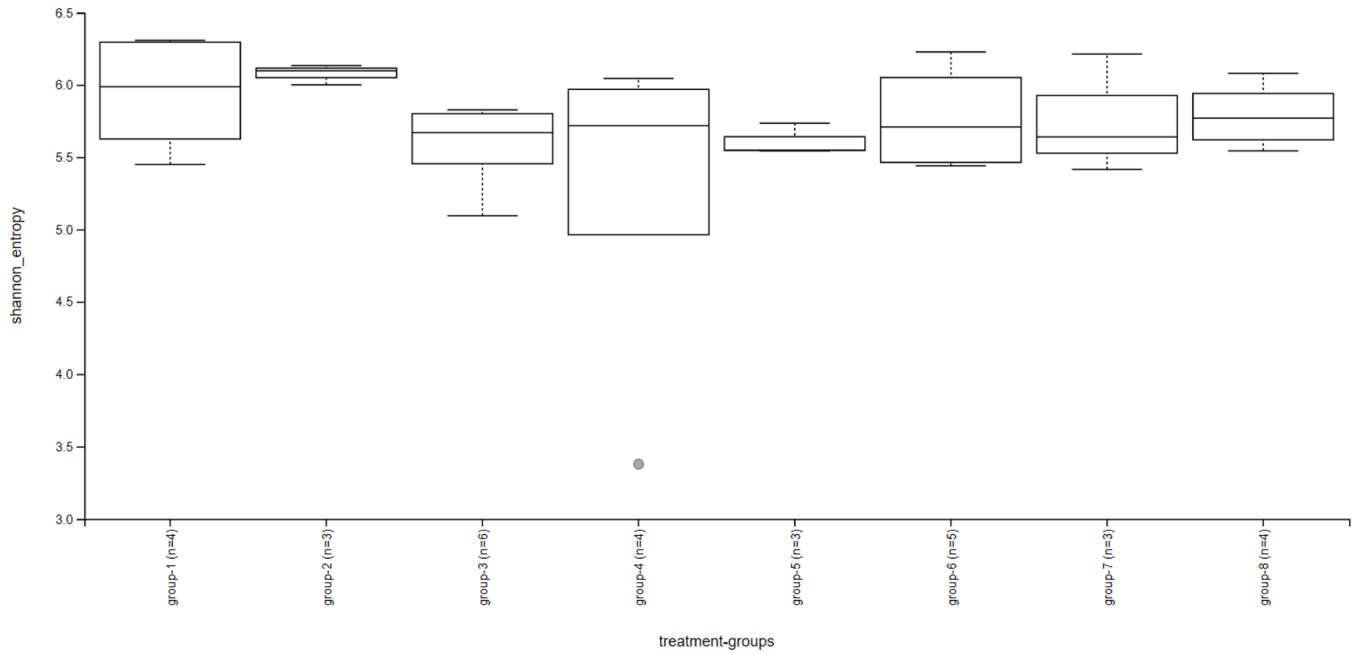

Fig. 1. Alpha diversity using the Shannon boxplots to view the microbial composition across all dietary treatments. Treatments include (group1-CD, group2-10 %FS, group3-1 %AS, group4-5 %AS, group5-10 %AS, group6-5 %APC, group7-10 %APC, group8-15 %APC. The significance level was determined using the Kruskal-Wallis statistical test fixed at $p < 0.05$.

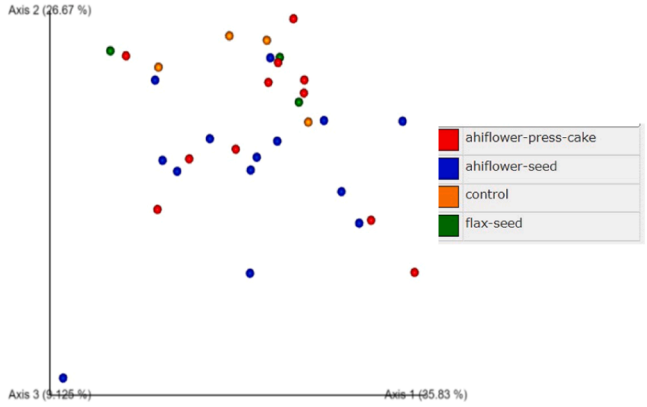

Fig. 2. PCoA plot to view the taxonomic classification among different dietary treatment using QIME.

with increased supplementation of flaxseed at 8, 12, and 16 %. Also, Lee et al. (1995) reported AME was reduced from dietary supplementation of 30 % flax seed in a laying hen trial. These differences can be attributed to the levels of flaxseed supplemented in the diets and the bird type in the different studies. The studies that used laying hens had younger birds, but our hens were older having more developed digestive system. We also observed that energy digestibility was reduced by 10 %APC compared to 10 %FS, 1 %AS, 10 %AS, and 15 % APC. The reduction would have been due to the low fat in the press cake after oil extraction from the seed and the high fiber content present in the press cake. However, it should be noted that the digestibility values reported in the current study were for the entire diet and not for specific ingredients, and other diet components contribute to these values.

The extent of Ca and P absorption and utilization depends on their dietary concentration which needs to be balanced to avoid negative effects on Ca digestibility (Anwar et al., 2018). In the current study, AS and APC were used to replace conventional Ca sources (limestone, oyster shell, and shell mix) thus all diets contained similar amounts of Ca and P, thereby saving cost of these expensive Ca sources. However, the study showed that Ca digestibility was significantly reduced in hens fed 10 % APC. While P digestibility in 10 %APC was like the control, it was lower than the 10 %FS, 1 and 10 %AS, and 15 %APC. This reduction in Ca and P digestibility in the 10 %APC diet could be a result of the high phytate and fiber contents in the APC (0.58 % P, 22.3 % NDF). Phosphorus may exist as phytate in bird feed ingredients causing low P availability (Marounek et al., 2008; Englmaierova et al., 2015). Furthermore, it has been well-documented that fiber and phytate could encapsulate nutrients, including minerals such as Ca and P, and reduce their digestibility (Gupta et al., 2015; Mirshekar et al., 2015). However, it is interesting to note that this reduction in Ca and P digestibility in some APC diets did not negatively affect the production performance and egg quality. This can be explained by the high Ca content in the APC (11.48 %), providing sufficient Ca that meets the hens' requirement despite its low digestibility.

Treatment-period interaction was observed such that 10 %FS reduced HDEP in period 1 and reduced FI in periods 1 and 2 without any effect in Period 3. It is possible that birds adjusted to the diet containing flax seed with time (Mridula et al., 2012). Novak and Scheideler (2001) observed that FI increased as birds age. The hens in the current study lost weight as they aged, which was reflected in the negative values obtained for BWG. It is common for hens to divert their energy and nutrients towards egg production rather than BWG towards the end of their production cycle (Aygun and Yetisir, 2013). The reduction in BWG by 10 % FS would have been caused by reduced feed intake due to the possible presence of antinutrients in FS. One of the antinutrients in flaxseed is trypsin inhibitors which bind with chymotrypsin and trypsin adversely affects the nutrient digestibility and growth rate of birds (Woyengo et al., 2017). Flaxseed also contains cyanogenic glycosides and phytic acid which impairs digestion and absorption thereby affecting the productivity of birds negatively (Ehr et al., 2017). Our observation agrees with Attia et al. (2022) who observed a reduction in the change in body weight in birds fed 12 % FS compared to control diet in 30week old Rhode Island Red laying hens. Also, Ehr et al. (2017) reported that single-comb white leghorn laying hens fed increasing levels of 2,3 and 5 % milled flax seed reduced change in body weight. Our findings are contrary to those of Al-Nasser et al. (2011), Yassein et al. (2015), and Huang et al. (2018a) who reported that 10 % FS did not affect birds'

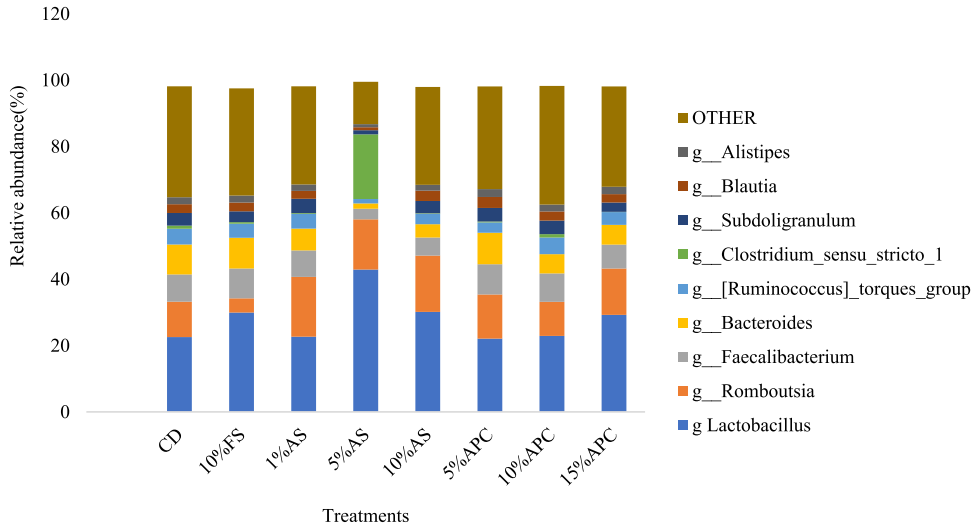

Fig. 3. Relative abundance of laying hens fed CD, FS (10 %), AS (1,5 and 10 %), and APC (5,10 and 15 %) diets.

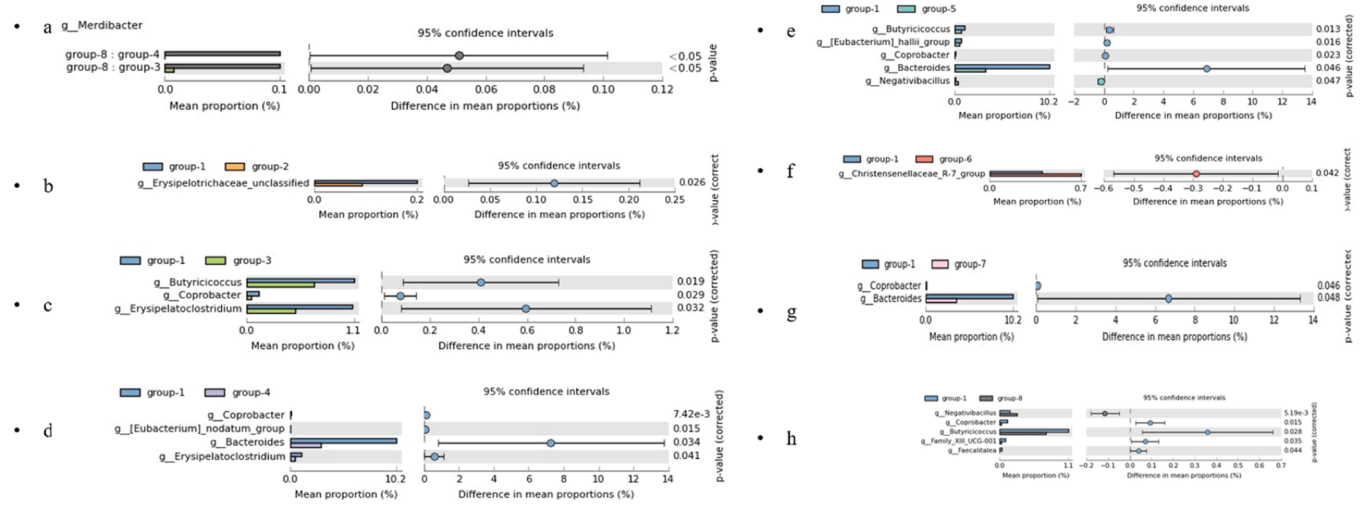

Fig. 4. Relative abundance of genus among dietary treatments using STAMP software package with the level of significance determined when $p < 0.05$. (a) level of significance of microbes in feces of hens fed 15 %APC and 1 % and 5 % AS. (b) level of significance of microbes in feces of hens fed 10 %FS and 1 % FS. (c) level of significance of microbes in feces of hens fed CD and 1 % AS. (d) level of significance of microbes in feces of hens fed CD and 5 % AS. (e) level of significance of microbes in feces of hens fed CD and 10 % AS. (f) level of significance of microbes in feces of hens fed CD and 5 % APC. (g) level of significance of microbes in feces of hens fed CD and 10 % APC. (h) level of significance of microbes in feces of hens fed CD and 15 % APC.

production performance. Novak and Scheideler (2001) reported that dietary supplementation of FS increased FI compared to control. Buğdayci et al. (2022) observed that 10 % ground or whole seed dietary supplementation of flaxseed did not affect FI and HDEP of quails which disagrees with our result. The difference in feed intake can be attributed to the dietary ME value. Birds feed to meet maintenance needs, and this has a relationship with the ME value of the diet (Harms et al., 2000). Huang et al. (2018a) reported no significant effect of dietary supplementation of flax at 7.5 % and 15 % on HDEP, FI, of 58-week-old hens compared to the control. However, Aziza et al. (2013) used 24-week-old hens, and Mridula et al. (2012) used 38-week-old hens in their study and reported that dietary supplementation of FS at 10 % increased HDEP compared to the control. Sari et al. (2002) reported that 10 % FS fed to 26-week-old hens reduced FI and FCR, but increased HDEP compared to control. These can be attributed to the difference in antinutritional composition in flax seed especially phytic acid which varies according to geographical location, planting season, and cultivars (Oomah et al., 1996). Furthermore, there seems to be a correlation between FI, BW, FCR, and HDEP which can be influenced by antinutrients present in flaxseed (Ehr et al., 2017).

El-Zenary et al. (2023) reported that 0.75 and 2.25 % ahiflower oil supplementation in the diet of hens did not affect HDEP, and FI of hens. In addition, El-Zenary et al. (2022) reported that dietary supplementation of ahiflower oil at 0.75 % and 2.25 % did not affect the FI of broilers. Ortiz et al. (2020) observed no significant difference in FI of broiler chickens from dietary supplementation of flaxseed at 40, 80, 160, and 240 g/kg. Furthermore, there seems to be a correlation between FI, FCR, and HDEP which can be influenced by antinutrients present in flaxseed (Ehr et al., 2017).

The treatments had a significant effect on the yolk color but not on the other egg quality parameters (egg weight, egg breaking strength, specific gravity, albumen height, shell weight, Haugh unit, or shell thickness). El-Zenary et al. (2023) reported that 22.5 % supplementation of ahiflower oil in the diet reduced egg weight compared to other treatments. This difference can be due to dietary ingredients such that El-Zenary et al. (2023) used pure ahiflower oil in their study while our study used whole ahiflower seeds and APC, which would have lower oil content and higher fiber content compared to the pure oil.

From our study, we observed that hens fed the 5 % APC had a lower yolk color L* compared to 10 % APC, as well as 10 % FS, and 15 %APC reduced a* color score compared to CD and 1 % AS as well as b* compared to 1 % AS and %APC. Internal egg quality such as egg yolk color is one of the egg quality parameters consumers evaluate (Saleh et al., 2021). There is variation in egg yolk color preference among people globally with orange yolk color being more preferred (Saleh et al., 2021). The egg yolk is in the range of pale yellow to dark orange color globally (Saleh et al., 2021). Yolk coloration is affected by factors such as the variety, quality, and amount of xanthophylls and carotenoids in diet, dietary fat, bird strain, feedstuff, age, and stress (Saleh et al., 2021). Corn has been reported to influence yolk pigmentation because it is rich in carotenoids (Kljak et al., 2021). Naturally, egg yolk is pigmented by xanthophylls, and carotenoids are present in diets, with xanthophylls as the major colorant (Lemahieu et al., 2014; Saleh et al., 2021). Carotenoids are essential in the diet of laying hens due to their inability to produce them and dietary xanthophylls must undergo saponification for them to be readily absorbed by birds (Saleh et al., 2021). In our study, the quantity of corn was reduced in the diet supplemented with FS, AS, and APC diets which could potentially affect yolk color. Yassein et al. (2015) and Aziza et al. (2013) reported that 10 % FS reduced yolk color in 40-week-old hens and 24-week-old hens compared to the control. Lemahieu et al. (2014) reported that the a* yolk color score increased while L* and b * values were reduced from dietary supplementation of 3 % *Isochrysis galbana* a microalgal rich in n-3 PUFAs. Although we did not determine the carotenoid level in yolk in this study, the reduction of L*, a*, and b* color scores could have been due to carotenoid pigmentation as the level and variety of dietary carotenoids can influence yolk color pigmentation (Lemahieu et al., 2014).

Dietary Ca and P concentrations influence the eggshell formation, quality, and breaking strength (Ahmad and Balander, 2003; Hassan and Aqil, 2015; Saki et al., 2019). In the current study, dietary treatments did not affect eggshells Ca and P levels. The high Ca (13.5 %,11.5 %) and P (0.55 % and 0.58 %) of AS and APC, respectively didn't affect eggshells Ca and P potentially due to the low digestibility of Ca and P, resulting from the presence of antinutritional factors such as phytate and non-starch polysaccharides which could bind Ca and P and prevent them from metabolized in the gut (Gupta et al., 2015). Laying hens are known to regulate Ca absorption especially when dietary Ca is high by reducing the amount of Ca absorbed into the blood (Clunies et al., 1992; Diana et al., 2021). Additionally, Plasma Ca and P levels would have influenced this occurrence, thereby limiting eggshell Ca and P levels (Clunies et al., 1992; Hunton, 2005; Gupta et al., 2015). Dietary Ca is absorbed into the blood after digestion of feed ingredients, after which nutrients including Ca pass through the blood for storage in the bones or go into the shell gland for eggshell formation (Hunton, 2005). Inorganic P dietary supplementation and the use of exogenous phytase have been reported to increase P concentration in the plasma and eggshell (Saban et al., 2005) and these are suggested approaches when ingredients with low Ca and P digestibility are utilized in laying hens' diets.

Dietary supplementation of FS, AS, and APC did not affect saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) composition in egg yolks due to the higher concentration of PUFAs from these feed ingredients. SFA causes the occurrence of heart disease by increasing low-density lipoprotein (LDL) cholesterol (Briggs et al., 2017). SFA can be categorized into medium-chain fatty acids (MCFA, consisting of 7-12 carbon atoms that are not saturated), short-chain fatty acids (SCFA, consisting of 1-6 carbon atoms that are not saturated), long-chain fatty acids (LCFA; consisting of more than 13 carbon atoms that are saturated or unsaturated). Examples of LCFA that are SFA include myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0). Increased consumption of MUFA and PUFA lowers LDL cholesterol in plasma (Briggs et al., 2017). MUFA have a single double bond and can have a cis or trans configuration depending on the hydrogen atom location on the double bond (Kazaz et al., 2022). MUFAs such as oleic acid has many health benefits such as the prevention of heart disease and suppression of tumor growth (Kazaz et al., 2022). Like our findings, El-Zenary et al. (2023) reported that dietary inclusion of 7.5 % and 22.5 % ahiflower and flax oil in laying hens did not affect SFA and MUFA egg yolks. El-Zenary et al. (2022) reported that the inclusion of 7.5 % and 22.5 % ahiflower oil and flax oil in broilers did not affect SFA and MUFA composition in the thigh but 7.5 % and 22.5 % significantly increased the SFA and MUFA composition in the breast. Furthermore, Yassein et al. (2015) reported that 10 %FS reduced total egg yolk SFA composition. In addition, Aziza et al. (2013) observed that 10 % FS reduced total egg yolk SFA and MUFA composition.

The n-3 FAs play anti-inflammatory roles in the human body as they produce different metabolites responsible for this action (Gheorghe et al., 2019). Both the n-3 and n-6 FAs compete for the enzymes responsible for desaturation into their varying metabolites which are the fatty acids desaturase enzymes (FADS) 1 and 2 (Simopoulos, 2016). These enzymes have a higher affinity for ALA than LA (Simopoulos, 2016). The inclusion of n-3 FA-rich feed ingredients in laying hen's diet for egg enrichment purposes has been adopted to improve n-3 FA consumption in humans for healthy living (Raza et al., 2016; Westbrook and Cherian, 2019). Dietary supplementation of 10 % FS and 10 % AS increased ($P<0.05$) total n-3 FA in egg yolks twice as much as was deposited in CD eggs. This is attributed to the increased concentration of ALA and its metabolites present in flax and ahiflower seeds, thereby increasing the bioavailability of ALA in these oilseeds. Similar to our observations, Al-Nasser et al. (2011) reported an increase in total n-3 FA in eggs from hens fed 10 % FS. However, Aziza et al. (2013) reported that total n-3 FA did not differ in egg yolk from hens fed 10 % FS compared to CD. There is a competition for desaturase and elongase enzymes by ALA and LA in poultry to produce VLC-FAs (El-Zenary et al., 2022). In our study, ALA deposit was higher in egg yolks from hens fed 10 % AS and 10 %FS compared to other treatments. This is attributed to the concentration of ALA (20.26 % and 9.91 %) in 10 % FS and 10 %AS diet, respectively, and it could be because of higher fatty acid digestibility and direct deposition of ALA from diet to yolk without undergoing elongation. This agrees with Mridula et al. (2012) and Perić and Drinić (2021) who reported that ALA concentration was increased in egg yolk from hens fed 10 %FS. ALA is desaturated and elongated to VLCn-3 with stearidonic acid as the first metabolite produced in the pathway; this reaction is catalyzed by FADS2 which is also involved in the reaction that yields DHA (Barceló-Coblijn and Murphy, 2009; Mirshekar et al., 2015). The deposition of SDA was higher ($P<0.05$) in eggs from hens fed 10 % AS which is expected due to the higher amount of SDA present in the ahiflower seed (0.86 mg/g) and diet (0.36 mg/g) compared to CD (amount in mg/g) and FS (amount in mg/g). This could also indicate a high bioavailability of SDA due to the micronization of AS. Micronization is a technique used in reducing the particle size of feed materials lower than ten microns to increase surface area thus enhancing digestibility, taste, and flavor (Dhiman and Prabhakar, 2021). Similarly, El-Zenary et al. (2022, 2023) observed an increase in SDA in egg yolks of laying hens, and thighs and breast of broilers from dietary inclusion of 22.5 g/kg of ahiflower oil. This agrees with Elkin et al. (2015) who reported that high SDA soybean oil at a 5 % inclusion level increased SDA deposit in egg yolks. In the current study, the dietary supplementation of AS at 5 % and 10 % and FS at 10 % increased the deposition of VLCn-3 PUFA, including EPA and DHA, in egg yolks. These observations agree with those of El-Zenary et al. (2023) who reported that there was a higher rate of VLCFAn-3 synthesis from ahiflower oil and flax oil supplementation. Elkin et al. (2015) stated that n6: n3 affects the synthesis of VLCFA from ALA, which explains why 10 % AS and FS (having lower n6: n3 than the other diets) had higher concentrations of VLCFAn-3 in the egg yolks compared to the rest of the treatments. EPA deposit in the yolk was increased by 10 % AS and 10 %FS diets and DHA was increased by 5 % and 10 %AS like 10 %FS. This indicates efficient absorption of VLC n-3 FAs, elongation, and desaturation of dietary SDA. The VLC n-3 FAs synthesis in the body depends on factors such as fatty acid desaturase (FADS) and elongase enzymes, competition for these

enzymes by substrate, and substrates (ALA, SDA, and LA) concentration (Barceló-Coblijn and Murphy, 2009; Mirshekar et al., 2015). Along the n-3 pathway, it has been observed that no competition exists with SDA for FADS2 therefore the increased deposition of VLCn-3FAs in yolks from AS supplementation which is rich in SDA (El-Zenary et al., 2022). El-Zenary et al. (2023) reported an increase in EPA from dietary supplementation of ahiflower oil at 0.75 % and 2.25 %. In our study, egg yolks from hens fed 10 % FS supplemented diet had reduced LA content. Studies have reported that LA concentration is reduced when there is an increase in n-3FAs deposit in the yolk (Elkin et al., 2015; Westbrook and Cherian, 2019; Perić and Drinić, 2021). Furthermore, in our study, the 10 %AS and 10 %FS supplemented diet had reduced AA and n-6: n-3 FA compared with other treatments indicating that ALA composition in the yolk increased. The n-6: n-3 FA ratio of 2.11 and 2.78 observed in our study from 10 %FS and 10 %AS is close to the recommended dietary ratio of 2:1 as stated by (Simopoulos, 2004). This may be due to the higher concentration of ALA in both ahiflower and flax seed which would have enhanced synthesis of VLCFA n-6 thereby reducing the n-6: n-3 ratio in yolks. ALA is the precursor required for the synthesis of VLCFA n-3 (Barceló-Coblijn and Murphy, 2009; Kihara, 2012; Gheorghe et al., 2019; Cartoni Mancinelli et al., 2022). In agreement, El-Zenary et al. (2023) reported that n-6:n-3 were reduced in egg yolks, breast, and thigh of broilers from birds fed diet containing ahiflower oil and flax seed oil at 2.25 % inclusion levels (Aziza et al., 2013; Westbrook and Cherian, 2019) reported that n-6: n-3 was reduced in egg yolks from 10 % FS. In our study, the egg yolks from hens fed at 15 %APC had the highest total n-6 deposit which can be due to the low-fat content present in press cake after oil extraction. The GLA levels were reduced in the egg yolks from hens fed 10 % FS compared to other treatments due to the low concentration of n-6 in flaxseed. Similarly, El-Zenary et al. (2022) reported that dietary supplementation of flax oil at 0.75 % and 2.25 % reduced GLA deposit in the yolk.

The composition of microbial communities in the gut can determine the health status of laying hens (Geier et al., 2009; Neijat et al., 2020). The microbial composition can be altered by factors such as diet nutrient digestibility, n-3 enrichment in diet, diseases, antibiotics, feed additives, and metabolic activities in the birds (Geier et al., 2009; Adewole and Akinyemi, 2021; Popescu et al., 2021). In the current study, treatment had no significant effect on the alpha diversity of fecal microbiota. This agrees with Neijat et al. (2020) who found that dietary supplementation of 0.20 % and 0.60 % flax oil, did not affect the cecal alpha diversity of laying hens. Similar findings were reported in 20-week-old laying hens' diets supplemented with 10 % flax seed (Mangi et al., 2021). On the contrary, Lee et al. (2015) reported that alpha diversity was affected by the dietary supplementation of 10 % flax oil in 36-week-old laying hens. The diet consisting of 10 % flaxseed will be high in fiber while the 10 % flax oil-supplemented diet will be high in fat. Fat and fiber differently influence gut microbiota composition such that microbiota composition changes based on their type and level in the diet (Machate et al., 2020; Cronin et al., 2021; Usuda et al., 2021). For instance, the ratio of *Bacteroides* to *Firmicutes* increased due to the consumption of a diet that is high in fatty acid compared to a lower fatty acid diet (Usuda et al., 2021). Also, gut microbial composition differs in birds based on their stage of development with older hens having a more stable microbial composition and more diversity (Grond et al., 2018; Ngunjiri et al., 2019; Sun et al., 2022).

At the phyla level, Firmicutes and Bacteroides were most abundant across treatments. Firmicutes secrete metabolites absorbed as an energy source by the gut wall and associated with an increase in chicken weight (Adewole and Akinyemi, 2021). Bacteroides aid in the digestion of food and nutrient absorption in the host gut (Pan et al., 2023). The increase in Firmicutes and Bacteroides abundance is an indicator of homeostasis activities to ensure the balance between short-chain fatty acid (SCFA) production and energy in the gut, thereby preventing intestinal inflammation (Thanabalan and Kiarie, 2021; Wu et al., 2022). The relative abundance of *Lactobacillus* was increased by dietary supplementation of 5 % AS. *Lactobacillus* is a beneficial bacteria belonging to the phylum Firmicutes. It aids in nutrient uptake (Neijat et al., 2020; Adewole and Akinyemi, 2021). The abundance of *Lactobacillus* can be influenced by n-3 PUFA (Geier et al., 2009). In an investigation by Geier et al. (2009) using salmate as the source of PUFA which contains 42 % fish oil and 58 % starch fed to broiler chickens at 2 % and 5 %, no effect of n-3 PUFA on the relative abundance of *Lactobacillus* on the digesta samples collected from the jejunum and ileum of the birds was observed. This difference can be attributed to bird type, dietary composition, PUFA source, and site of sample collection from the birds.

The abundance of *Merdibacter* was significantly higher in the feces of hens fed 15 % APC compared to 1 % and 5 % AS. *Merdibacter* plays a role in metabolic homeostasis in the gut of humans due to its metabolic ability to harvest dietary nutrients and energy. The abundance of *Merdibacter* in the feces of hens fed 15 % APC diets could relate to energy absorption by the host due to the high fiber in the diet (the 15 % APC diet consist of 11.3 % NDF, while 8.34 and 8.21 % NDF were present in the 1 and 5 % APC diets, respectively), which can increase SCFA production (Hou et al., 2020). We observed that the relative abundance of *Erysipeiotrichaceae_unclassified* was significantly reduced when hens were fed 10 % FS. *Erysipeiotrichaceae_unclassified* has been identified as one of the bacteria that are harmful to the host (Li et al., 2024). *Erysipeiotrichaceae-unclassified* abundance could have been reduced due to the prebiotic properties of flaxseed, causing an increased abundance of beneficial bacteria in the gut which potentially compete with the harmful ones, thus reducing their abundance (Mueed et al., 2022). We also observed that *Butyricicoccus, Coprobacter*, and *Erysipelatoclostridium* were significantly reduced by dietary supplementation of 1 % AS. Furthermore, *Bacteroides* and *Erysipelatoclostridium* were reduced by dietary supplementation of 5 % AS. In addition, dietary supplementation of the 10 % AS reduced *Butyricicoccus, Coprobacter, Erysipelatoclostridium, Eubacterium, Coprobacter*, and *Bacteroides* abundance, while dietary supplementation of 15 % APC reduced the abundance of Butyricicocus, *Coprobacter* all in comparison to CD. These various microbes play essential roles in the gut such as SCFA production. *Butyricicoccus* belongs to the family Ruminococcaceae and can secrete butyrate, which is an energy source in the gut (Zhang et al., 2021). *Coprobacter* belongs to the family Rikenellaceae and secretes SCFA such as acetate (Sakamoto et al., 2021; Mann et al., 2024). Acetate plays a role in energy production in the gut (Mann et al., 2024). *Erysipelatoclostridium* is associated with a high-fat diet resulting in the occurrence of obesity in humans (Ye et al., 2021). Meanwhile, *Eubacterium* belongs to the phylum Firmicutes, and their abundance is reduced with increased dietary fat or protein composition (Mukherjee et al., 2020). *Eubacterium* are high SCFA producers and their abundance correlates positively with SCFA production in the gut (Mukherjee et al., 2020). Diets high in fat reduce the production of SCFA. *Bacteroides* are involved in producing SCFA that serves as a source of energy for their host (Wexler, 2007). The reduction in the abundance of the different bacteria involved in the production of SCFA by dietary supplementation of AS at various levels in this study can be attributed to the high concentration of n-3 PUFA in these diets fed to the hens. PUFA has been reported to inhibit the production of SCFA in the gut (Neijat et al., 2020). Also, the total non-starch polysaccharides (NSP) content in AS (18 %) is lower than that of APC (23.2 %) thereby limiting the proliferation of SCFA bacteria to break down polysaccharides (Onrust et al., 2015). Lee et al. (2015) reported that *Bacteroides* abundance was reduced from the dietary supplementation of flax oil which is rich in PUFA at 5 % and 10 %. Furthermore, 10 % AS reduced the abundance of *negativiballus* while 15 % APC increased its abundance. In a study carried out using human feces Mai et al. (2024) observed that *negativiballus* was abundant in feces collected from patients diagnosed with non-alcoholic fatty liver disease (NAFLD). They predicted that NAFLD occurrence would progress when *negativiballus* is in abundance thereby upregulating lipopolysaccharides glutathione and lipid metabolic activities. From our observation, the

reduction and increase of *negativiballus* by 10 %AS and 15 %APC, respectively can be attributed to the dietary n-3 PUFA composition and NSP content as 10 %AS which has a higher composition of n-3 PUFA but a lower content of NSP than 15 %APC. The n-3 PUFA plays an anti-inflammatory role in the body by reducing the level of arachidonic acid thereby inhibiting the inflammatory action of n-6 PUFA and its derivatives (Geier et al., 2009; Al-Khalifa et al., 2012; Gheorghe et al., 2019; Neijat et al., 2020). The NSP content of AS (18 %) is lower than APC (23 %). Dietary NSP level (depending on its components) could be a concern in the poultry industry as soluble NSPs could cause the growth of harmful bacteria in the gut resulting in the occurrence of infectious enteric diseases which could have led to the proliferation of *negativiballus* (Nguyen et al., 2022).

We also observed that the abundance of *Christensenellaceace R-7_group* was increased when hens were fed a 5 %APC diet. *Christensenellacea R-7_group* belongs to the phylum *Firmicutes* and ensures that the energy demand of host organisms is met by aiding in the release of energy from the adipose tissue. The abundance of *Christensenellaceae_R-7_group* could have increased to make energy available to the birds from stored fat in the adipose tissue due to the low-fat composition of the 5 % APC diet after oil extraction from the seed. Furthermore, dietary fiber is a source of SCFA through microbial fermentation and improves the gut microbial community (Hou et al., 2020). The APC diet has a high NDF which would have increased the abundance of *Christensenellaceace R-7_group* to ferment the fiber into SCFA to make energy available to the birds. Furthermore, we observed that dietary supplementation of 15 % APC reduced the abundance of *Family_XIII_UCG-001, Faecalitalea.* The abundance of *Family_XIII_UCG-001* has been associated with the occurrence of hypertension and coronary artery disease due to a rise in low-density lipoprotein cholesterol, serum cholesterol, and triglycerides (Huangfu et al., 2021) while Montgomery et al. (2024) reported that an abundance of *Faecalitalea is* associated with an unhealthy state of the gut as well as it causes constipation. The reduction of this bacteria can be attributed to the increase in butyrate production caused by the high dietary fiber. Butyrate helps maintain intestinal integrity and plays anti-inflammatory roles in broiler chickens. In a study by Zou et al. (2019) using sodium butyrate (SB) as a dietary supplement, it was observed that SB reduced hemorrhage and lesions in the gut. The result shows that dietary supplementation of AS and APC at different inclusion levels may modulate microbial composition differently due to the differences in their fat and fiber compositions.

Conclusions

In conclusion, dietary supplementation of ahiflower seed at 10 % increased n-3 FAs and reduced n-6: n-3 FAs in egg yolks, in the same capacity as 10 %FS. All press cake inclusion levels did not affect n-3 FAs while 15 %APC increased total n-6 FAs. Also, dietary inclusion of AS up to 10 % and APC up to 15 % did not influence FCR, and eggshell Ca and P levels negatively, but 10 %FS negatively influenced BWG, HDEP and FI. The yolk color lightness, redness, and yellowness were reduced by 5 and 15 % APC and 10 %FS. Furthermore, energy digestibility in laying hens was improved by 10 %FS, 1 %AS and 15 %APC while AME and AME_n was increased by 10 %FS and all levels of AS and APC inclusions. The digestibility of P was not negatively affected by any of the inclusion levels of AS and APC; however, Ca digestibility was reduced by 10 % APC, compared to the control. The inclusion of AS and APC in laying hens' diets favorably offsets the need for traditional Ca supplementation as it reduces the inclusion of other sources of Ca in the diet, maintaining eggshell quality and improving overall hen-day egg production vs the CD diet, while not inhibiting other production performance parameters. All AS and APC levels influenced the microbial community due to their PUFA, dietary fiber, and NSP compositions. While all AS and APC levels increased the abundance of bacteria involved in SCFA production, the 15 %APC increased the proliferation of a harmful bacteria (*negativiballus)* but reduced some other harmful bacteria (*butyricicoccus, coprobacter, family_XIII_UCG-001, faecalitalea)* in the feces.

Disclosures

The authors declare the following conflict of interest: Greg Cumberford is one of the authors and is also a staff member of Natures Crops International, one of the organizations that funded this project.

CRediT authorship contribution statement

Roseline O. Ogory: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Greg Cumberford:** Conceptualization, Writing – review & editing. **Deborah Adewole:** Conceptualization, Data curation, Writing – review & editing.

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